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Clozapine-Induced Cell Growth Inhibition of *Saccharomyces cerevisiae*

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Clozapine-Induced Cell Growth Inhibition of *Saccharomyces cerevisiae*

Major Qualifying Project Report for Worcester Polytechnic Institute

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Abstract

Clozapine, a current schizophrenic medication, can adversely affect a patient's immune system. Clozapine causes agranulocytosis (loss of white blood cells) in some patients. The mechanism underlying clozapine-induced agranulocytosis is not well understood. Our working hypothesis is that clozapine reacts with hydrogen peroxide (H_2O_2) within granulocytes, generating a toxic product that kills the immune cells. We have developed an assay in the model organism – *Saccharomyces cerevisiae* to understand the effects of clozapine. We produced 11 reproducibly repeated trials that resulted in a negative correlation between the concentration of clozapine and yeast cell density. The establishment of a reliable assay for clozapine-induced cell death and the determination of the MIC_{50} of clozapine for yeast cells may contribute to future *S. cerevisiae* genomic screens, and the repeated dose dependent inhibition of yeast cell growth by clozapine, in the presence of H_2O_2 , indicates that yeast cells can indeed serve as a model for understanding agranulocytosis in the population of patients treated with clozapine.

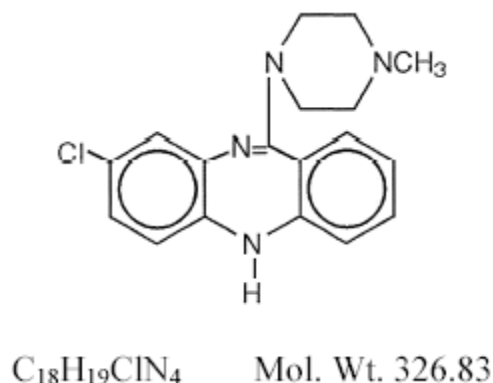
Introduction

Rationale and Project Summary

Clozapine is currently the most effective drug for treating schizophrenia, which is a disease with pervasive impacts on our society. Clozapine is especially important as the drug of choice when treating refractory (resistant to treatment) schizophrenia, which represents one-quarter of the total schizophrenia diagnoses. In addition, clozapine is currently being researched for its potential role in treating other disorders, such as psychotic symptoms occurring in patients with dementia of the Lewy-body-type, intractable chronic insomnia, and schizoid personality disorder. Unfortunately, clozapine has been relegated to third-line use, even though it could be more effective than other drugs, due to its potentially lethal side effects, mainly agranulocytosis and myocarditis. Although the mechanism of clozapine-induced agranulocytosis is still unknown there are two main hypotheses and these are the immune and toxic mechanisms. The toxic mechanism hypothesis proposes that some toxic product or aspect of clozapine itself causes the death of white blood cells. Whereas, the immune hypothesis proposes that it is the interaction between the H_2O_2 produced by white blood cells and clozapine that then kills white blood cells. Researchers are trying to understand the mechanism in hopes of producing a safer version of clozapine that does not cause agranulocytosis. (Wahlbeck, 2007)

The clozapine mechanism of symptom relief is proposed to be preferential binding to serotonin 5-HT₂ and dopamine D₄ receptors relative to dopamine D₂ receptors, (Sharafi, 2005). Clozapine, which was first introduced in 1971, is a tricyclic dibenzodiazepine derivative, 8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo [1,4] diazepine; Figure 1 shows its structural formula, (WebMD, 2009).

Figure 1. The Structural Formula of Clozapine



There are several other atypical antipsychotics, but clozapine is by far the best at bringing about symptom relief to the large percentage of sufferers of refractory schizophrenia. In fact, clozapine is often the only drug that brings any relief at all to these patients. Clozapine is more effective than other antipsychotic drugs in reducing the positive symptoms and the harder to treat negative symptoms of schizophrenia, eliminating the dyscognitive effect, motor adverse effects and extrapyramidal side effects seen with other psychoactive drugs, fostering social rehabilitation, reducing the propensity for substance abuse, prolonging the time to suicidal attempt, and producing clinically meaningful improvements and postponing relapse, (Lee, 1998 and McEvoy, 2006). This may explain why patients are generally more satisfied with clozapine treatment than with typical antipsychotic treatment. (Wahlbeck, 2007)

Due to the fact that it is known to cause agranulocytosis in approximately 1% of patients, clozapine was restricted entirely by the US Food and Drug Administration (FDA) for a period of time, and is currently only allowed for treating patients as a last resort. Even when clozapine is allowed for treatment after meeting the strict guidelines for the last resort drug, the patient must undergo weekly mandated blood tests to detect leukopenia; this can prove difficult or impossible for severely schizophrenic patients and their families to adhere to. Ultimately, refractory schizophrenics are left with no satisfactory solution, and patients unable to qualify for clozapine are being treated with drugs that may not be providing them with as effective symptom relief as clozapine could.

Establishing that clozapine and its mechanism of white blood cell death can be studied in the model organism *S. cerevisiae*, would allow for improved and expanded research to determine the mechanism of action behind clozapine-induced white blood cell death. Being able to study clozapine and agranulocytosis in a system other than in-vitro agranulocytes is especially significant when that system is *S. cerevisiae*, since its entire genome has been sequenced and novel experiments could be conducted that would be impossible with agranulocytes. If clozapine's negative side-effects, particularly agranulocytosis, could be understood and overcome, this effective antipsychotic could provide much needed relief to millions of schizophrenic patients.

Background on Schizophrenia

Schizophrenia, one of the most widespread psychiatric disorders, is a chronic, severe, and disabling brain disorder. It is defined as a psychiatric diagnosis that describes a mental disorder characterized by abnormalities in the perception or expression of reality. (American Psychiatric Association, 2000) Simply put, schizophrenia causes patients to lose touch with reality, because they are often unable to make sense of the signals they receive from the world around them. Schizophrenia is marked by severely impaired reasoning and emotional instability, and it can often cause violent behavior. Schizophrenics often believe people, objects and events to be very different from what they really are. If untreated or refractory to treatment, most schizophrenics gradually withdraw from the outside world or are unable to function within it. (Tsuang, 1997)

Prevalence and Impact

Schizophrenia is truly a debilitating disease that effects many people and is a major public health problem with pervasive impact on our society. It affects approximately 24 million people worldwide and more than two million Americans in a given year, (Sharafi, 2005). Estimates reach as high as 1% of the world's population being affected, (Tsuang, 1997). A 2002 systematic review of 18 prevalence and eight incidence studies, which met stringent eligibility criteria for the review, found a lifetime prevalence rate of 0.55%, (Goldner, 2002). Approximately, half of all patients in psychiatric hospitals are diagnosed with schizophrenia and they may occupy as many as one quarter of the world's hospital beds, (Tsuang, 1997). Not only are the many sufferers of schizophrenia affected, but their families are as well. Families of schizophrenics struggle with a sense of loss and the demanding lifestyle imposed by caring for loved ones dealing with the disorder. Even books and movies, such as "A Beautiful Mind," have portrayed the often devastating cultural effects of schizophrenia. (Pescosolido, 1999)

Epidemiology

Schizophrenia affects people of all ages, races, genders, social classes, levels of education, or ethnic backgrounds, (Tsuang, 1997). It occurs equally in males and females, although it typically appears earlier in men. The peak ages of onset are 20–28 years for males and 26–32 years for females. (Castel, 1991) Most patients are diagnosed in their late teens or early twenties, but the disorder can appear at any time in a person's life and it has even been reported in children as young as five years of age, (Tsuang, 1997). It also occurs all over the world at similar rates. However, the prevalence of schizophrenia does vary among countries, within them and at the local level. (Kirkbride, 2006) For example, interesting epidemiology studies have indicated a correlation between urban environments and schizophrenia even after controlling for factors such as drug use, ethnic group and size of social group, (Van Os, 2004).

Symptoms and Prognosis

Schizophrenia's main debilitating effect is the deterioration of proper cognition, or information processing. It also usually contributes to chronic problems with behavior and emotion, such as paranoia, avolition (lack of motivation), and apathy. Patients exhibit lifelong deficits with the majority of symptoms described by 'the four As':

- 1.) Association disturbances
- 2.) Ambivalence
- 3.) Affect disturbances
- 4.) Autism

It was Eugen Bleuler, an influential Swiss psychiatrist, who in 1908 proposed the four A's to describe the main symptoms of schizophrenia and in 1911 introduced the term schizophrenia to replace dementia praecox. (Kuhn, 2004) There are five recognized subtypes of schizophrenia: paranoid, disorganized, catatonic, undifferentiated, and residual. Schizophrenia symptoms are very hard to define and many categories of symptoms are used in diagnosis and treatment. (Tsuang, 2000) However, the two main categories are positive and negative symptoms. Positive symptoms are forms of psychosis and include delusions, auditory hallucinations, and thought disorder. Thought disorder may encompass tangible symptoms such as, having trouble completing a sentence, thinking through an idea, answering a question clearly, or carrying out routine tasks. An interestingly common and severe symptom is called insertion or withdrawal of thought, which refers to the patient's misconception that someone or something can put thoughts in the patient's head or take them out. (Tsuang, 1997) Negative symptoms, which are more common, include flat or blunted affect (the lack of emotion), alogia (poor speech abilities), anhedonia (the inability to experience pleasure), and avolition, (Tsuang, 1997).

Schizophrenics are likely to have comorbid conditions, including major depression and anxiety disorders, (Sim, 2006). Schizophrenia also increases the lifetime substance abuse prevalence from 17% in the general U.S. population (Sussman, 2008) to around 40% in the population of schizophrenics (Friedman, 2000). Schizophrenia leads to many social problems, including long-term unemployment, poverty and homelessness, and schizophrenics are often unable to function independently and may become a burden to their families or require hospitalization. (Friedman, 2000) There is a higher than average suicide rate associated with schizophrenia which has been cited as high as 10%, although recent analysis of studies and statistics revises the estimate at 4.9%, (Palmer, 2005). In addition, many of the antipsychotics have unhealthy side effects, such as weight gain and increased prevalence to diabetes. This combined with the higher suicide rate in schizophrenics, has led to the average life expectancy of people with the disorder being ten to 12 years less than those without schizophrenia. (Wahlbeck, 2007)

Causes

The cause of schizophrenia remains unknown and has been the subject of debate for centuries. Studies suggest that genetics, early environment, neurobiology, psychological and social processes are important contributory factors. Some recreational and prescription drugs, including cocaine and amphetamines, appear to cause or worsen symptoms. (Friedman, 2000) Current psychiatric research is

focused on the role of neurobiology, but no single organic cause schizophrenia has been found. (Susser, 2002)

For much of the twentieth century, scientists thought that stressful or traumatic conditions (such as abuse) in a person's life could cause mental disorders. (Friedman, 2000) This theory is now less popular with scientists, who generally agree that the disease is biological and not caused by life experience. For example, research shows that the condition tends to run in families. A person with schizophrenic relatives is ten times as likely to develop schizophrenia as someone who has no history of the disease in the family. (Tsuang, 1997)

There is evidence to support NRG1 and DTNBP1 as schizophrenia susceptibility loci, although further research is required to understand how genetic variation at each locus confers susceptibility and protection. (O'Donovan, 2003) Also, some researchers have argued that schizophrenia is caused by a virus that attacks the brain. However, the most widely accepted theory is that schizophrenia is caused by an imbalance of neurotransmitters in the brain. (Patterson, 2006) This influential theory, known as the dopamine hypothesis of schizophrenia, proposed that excess activation of D₂ dopamine receptors was the cause of the positive symptoms of schizophrenia. The hypothesis was originally based primarily on the accidental finding that a drug group which blocks dopamine function, known as the phenothiazines, could reduce psychotic symptoms and the fact that amphetamines, which trigger the release of dopamine, may exacerbate the psychotic symptoms in schizophrenia. (Laruelle, 1996)

Brain imaging technology, such as fMRI, CT, and PET, has shown that there are physical differences in the brains of schizophrenics, which supports the idea that the cause of schizophrenia is biological. Studies supporting the dopamine hypothesis have shown that the functional differences in brain activity between normal and schizophrenic brains seem to most commonly occur in the frontal lobes (an area of the brain which contains most of the dopamine-sensitive neurons in the cerebral cortex), hippocampus and temporal lobes. (Kircher, 2006) Still, there is no consensus as to which, if any, of these theories is correct. It could be very possible that the disease is caused by a combination of factors.

Diagnosis

Since there is no specific cause of schizophrenia, it is diagnosed on the basis of symptom profiles. Psychiatric assessment includes a psychiatric history and some form of mental status examination. (American Psychiatric Association, 2000) Exactly what schizophrenia is, and should be defined as, has been the source of considerable disagreement among psychiatrists. Due to the many possible combinations of symptoms, there is even debate about whether the diagnosis represents a single disorder or a number of discrete syndromes. (Friedman, 2000) This is why Eugen Bleuler actually termed the disease the schizophrenias, when he coined the name, to emphasize the appearance of several discrete syndromes, (Kuhn, 2004).

There are currently no laboratory tests by which schizophrenia can be diagnosed. However, some imaging techniques can be helpful in showing abnormal structures in the brain. For example, individuals with schizophrenia, including those who have never been treated, typically have enlarged ventricles in the brain, as demonstrated in over 100 studies to date. They have a reduced volume of gray matter (up to 25% in some areas) in the brain, especially in the temporal and frontal lobes. (Barrow, 2008) Some

studies have reported that about half of the chronic cases of schizophrenia were also shown to exhibit hypofrontality in the brain at rest, a condition in which strong associations with the negative symptoms of schizophrenia have been reported, (Sharafi, 2005). For the most part, however, doctors must observe a patient's behavior to decide if he or she is schizophrenic, and to rule out other physical and mental disorders with similar symptoms, such as encephalitis, (American Psychiatric Association, 2000).

Refractory Schizophrenia

Refractory, or treatment-resistant, schizophrenia is defined in practice as the failure of symptoms to respond satisfactorily to at least two different antipsychotics. However, the precise definition is still controversial. (Meltzer, 1997) Approximately 30% (range 10-45%) of schizophrenic patients meet the criteria for refractory schizophrenia, according to at least two reports, (Meltzer, 1997 and Bressan, 2007). Although some averages are slightly lower at 25%, (Meltzer, 1997). Of the remaining 70% of schizophrenic patients whose positive symptoms respond adequately to antipsychotic treatment, many may still have clinically significant negative symptoms and cognitive function, and poor social and work function. This can mean that although these patients no longer experience severe positive symptoms they still suffer poor quality of life relative to the normal population and/or continue to constitute a significant burden to family and society.

Since refractory schizophrenics exhibit severe symptoms unique to this condition which lead to more frequent and longer duration hospitalizations, their treatment is generally much more expensive than that of neuroleptic-responsive patients. They usually have poorer premorbid function, an earlier age at onset of positive symptoms, and more severe cognitive dysfunction. Even magnetic resonance imaging scans show that these patients have features unique from other schizophrenics, such as various quantitative types of cortical or ventricular abnormalities. Commonly used techniques for inducing responsiveness, such as increased dosages of neuroleptic drugs, switching to other types of neuroleptics, or adding adjunctive agents such as antidepressants or anticonvulsants does not work in refractory schizophrenics. (Meltzer, 1997) It is clear that refractory schizophrenia represents a challenge to clinicians and that it is often under diagnosed and undertreated. (Bressan, 2007)

Treatment

The primary form of treatment for schizophrenia is medication, and drugs are now available to control many of the symptoms of the disorder. Most of these medications alleviate the symptoms, but never 'cure' the disease. (Barrow, 2008) Between 60 to 70 percent of patients with schizophrenia respond to drug treatment. The most successful medications used in the treatment of schizophrenia are neurotransmitter antagonists. Schizophrenia is a complicated disorder with many different forms of manifestation. In accord with logic, patients do not only manifest different forms of schizophrenia but they also respond differently to different treatments. (Patterson, 2006) This is in part the reason for great value in having as many different effective medications as possible. Although most researchers believe that schizophrenia is a biological problem rather than a problem caused by early upbringing or

life events, psychotherapy, including counseling and behavior therapy, is often part of the treatment plan for a schizophrenic. (Friedman, 2000)

Drugs for Treating Schizophrenia

There are two classes of antipsychotics: typical, or first generation, and atypical, or second generation, antipsychotics. Typical antipsychotics were first introduced in the 1950s and are divided into high-potency, fluphenazine and haloperidol, and low-potency, chlorpromazine, drugs. These drugs are known to have many symptoms, the most notable of which are the extrapyramidal side effects (EPS). (Wang, 2005) EPS is characterized by tremor, slurred speech, akathisia (a movement disorder characterized by inner restlessness and the inability to sit or stand still), dystonia (a neurological movement disorder characterized by involuntary muscle contractions), bradyphrenia (a slowing of thought processes), and bradykinesia (a slowing of movement and muscular rigidity). The side effects resemble the signs of Parkinson's disease, which are associated with degeneration of the dopamine nerve tracks located in the extrapyramidal region of the central nervous system. (Weiden, 2007) In addition, with typical antipsychotics there is a significant risk of the serious condition tardive dyskinesia, which develops in approximately 30 percent of the patients, (Llorca, 2002). Another serious side effect that is most common amongst the typical antipsychotics is neuroleptic malignant syndrome (NMS), a life-threatening neurological disorder. (Ananth, 2004) Long-term treatment of schizophrenia with typical antipsychotics has limitations, since it results in 25 to 30 percent of the patients showing treatment-resistance. (Wahlbeck, 2007)

Atypical antipsychotics, such as clozapine, are actually a group of unrelated drugs that are grouped together simply because they work differently from typical antipsychotics. Both typical and atypical antipsychotics block dopamine D₂ receptors and signal transmissions by dopamine. This mechanism of symptom relief is in accord with the dopamine hypothesis that schizophrenia is associated with increased activity in dopaminergic neurons. (Sharafi, 2005) However, atypicals are different from typical antipsychotics in their mechanism of action since most act on serotonin receptors as well as dopamine receptors, (Seeman, 2002). The first atypical anti-psychotic medication, clozapine, was introduced in the 1970s. Next, olanzapine, risperidone and quetiapine were introduced in the 1990s, and ziprasidone and aripiprazole followed in the early 2000s. Paliperidone is the latest atypical to be approved by the FDA in 2006. (Weiden, 2007) The most strikingly different clinical effect of atypical drugs is their decreased propensity to cause EPS, (Farah, 2005). The definition of an atypical antipsychotic was based upon the absence of EPS found in clozapine, the first atypical antipsychotic. The atypical drugs that followed clozapine have now been shown to still induce EPS, although to a lesser degree than typical antipsychotics. Since all of the post-clozapine atypical antipsychotics still affect the dopamine D₂ receptor, the continued presence of EPS is consistent with its hypothesized mechanism. (Weiden, 2007) But fewer patients will get EPS at therapeutic doses of one of the atypical antipsychotics than the typical antipsychotics, and when EPS do occur, they tend to be less severe. Therefore, most researchers agree that atypical antipsychotics, in general, have lower EPS liabilities.

Clozapine, an atypical antipsychotic, is more effective in treating schizophrenia than typical antipsychotics and may help to reduce relapses, suicide and necessary hospitalization, as demonstrated

in a 2005 comparison study. The study combined neuropsychological evaluation and positive and negative syndrome scale of schizophrenia (PANSS) scores, with single photon emission computed tomography (SPECT) to detect differences in the effectiveness of clozapine and typical antipsychotics. After treatment, different brain focal abnormalities in the two groups were observed and differences in PANSS scores were significant in both groups, with superior scores resulting from treatment with clozapine. Results were supported by SPECT, which showed a greater improvement in the clozapine group. Both positive and negative symptoms were improved with clozapine as well. Before treatment, hypofrontality, which is strongly associated with negative symptoms, occurred in 85% of the patient's brains and most cases of hypofrontality were cleared after treatment with clozapine. An interesting finding in the study may explain why clozapine is more effective, especially with refractory schizophrenia; there was a significantly increased rCBF observed in the thalamus that was more prominent in clozapine-treated patients, which could be due to clozapine's greater effect on the thalamus by dopaminergic receptors. (Sharafi, 2005)

Although there is some evidence that non-clozapine atypical antipsychotics are more advantageous than typical drugs, clozapine is superior to these other atypical drugs as well. Clozapine is known to have the fewest EPS of all antipsychotics, including the other atypicals, (Weiden, 2007). According to a systematic review in 2003 comparing the EPS in atypical antipsychotics and low-potency typical antipsychotics, only clozapine was associated with significantly fewer EPS (RD=-0.15, 95% CI -0.26 to -0.4, p=0.008) and higher efficacy than low-potency typical drugs, (Leucht, 2003). According to a trial conducted by Jones in 2006, there is no disadvantage across one year in terms of quality of life, symptoms, or associated costs of care in using typical versus non-clozapine atypical antipsychotics. It is important to note that the superiority of clozapine in this trial was neither the result of inadequate power nor patterns of drug discontinuation. (Jones, 2006) In addition, clozapine is the only drug that is consistently effective in the population of treatment-refractory schizophrenics. In 2000, David Taylor developed a refractoriness rating based on previous work evaluating other atypicals as treatments for refractory schizophrenia. The goal was to evaluate the current evidence of effectiveness of other atypical antipsychotics in comparison to clozapine. He did this by assessing all trials of atypical drugs in schizophrenia unresponsive to at least one drug with the refractoriness rating he developed. His analysis determined that clozapine should remain the drug of choice to treat refractory schizophrenia, since there was no evidence to support the use of any of the other atypical drugs. Even when using stringently defined refractoriness ratings, clozapine was consistently shown to be more effective. (Taylor, 2000)

Unfortunately, clozapine has several adverse effects that do extend beyond agranulocytosis, which is the adverse effect focused on in this study. Clozapine must carry five black box warnings for agranulocytosis, seizures, myocarditis, other adverse cardiovascular and respiratory effects, and increased mortality in elderly patients with dementia-related psychosis. Myocarditis usually develops after approximately one month of starting the drug and it presents with obvious signs of illness. However, cardiomyopathy is also potentially life threatening and could arise suddenly or go undetected. Consequently, a regular six-monthly echocardiogram was recently recommended when treating a patient with clozapine. (Haas, 2007) Clozapine is also known to cause gastrointestinal hypomotility, (Palmer, 2008) and deficiency of selenium, (Vaddadi, 2003). Available data indicates that clozapine and olanzapine are associated with the greatest effects on weight gain and decreased insulin sensitivity,

followed by risperidone and quetiapine. Therefore, clozapine (along with other atypical antipsychotics) results in increased susceptibility to weight gain and obesity related illnesses, particularly diabetes, and may increase this susceptibility more than most typical drugs. (American Diabetes Association, 2004) However, the primary risk with clozapine and the primary factor relegating clozapine to its status as a last resort drug remains agranulocytosis. Eliminating this lethal side effect would make clozapine much more relatively safe in comparison to the other antipsychotics.

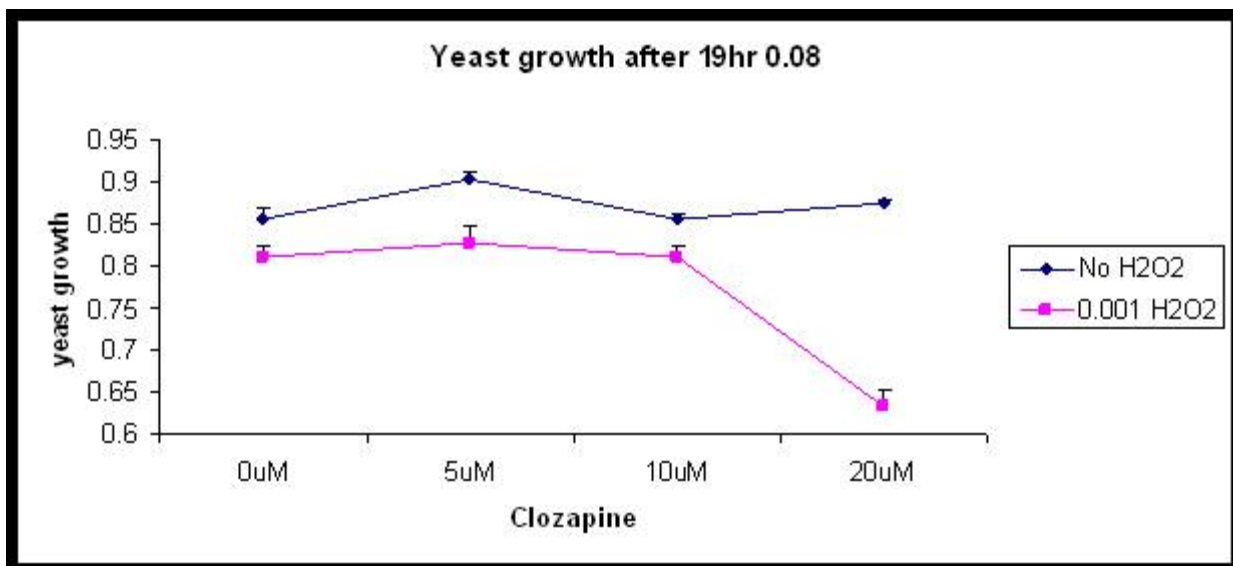
Materials and Methods

Developing the assay for clozapine-induced yeast cell growth inhibition

In order to determine if clozapine does indeed cause inhibition of growth in yeast cells, many liquid yeast cultures of the *S. cerevisiae* Y101 yeast strain were experimented with under different reaction mixture conditions. Different amounts of yeast, clozapine, HRP, H₂O₂ and DMSO were combined and the cellular density was determined after zero, eight and 12 hours of culture. Educated trial and error was necessary to determine if some specific combination of different concentrations of each variable would result in an assay that does show dosage dependent inhibition of yeast cell growth by clozapine. Eventually, this goal was reached and a total of 11 trials with Y101, clozapine and H₂O₂ resulted in dosage dependent inhibition of cell growth. Each 96-well plate trial contained duplicates of each reaction mixture, meaning that a total of 22 sets of reaction mixtures at different concentrations of clozapine and H₂O₂ showed that clozapine does negatively affect cell growth.

The inspiration for this project came from Dr. Abraham Kovoov's lab (University of Rhode Island). They worked to show that yeast cell growth is negatively affected by clozapine, in the presence of H₂O₂, and could therefore possibly serve as a model for investigating clozapine-induced white blood cell death. They were able to produce a trial that resulted in dosage dependent yeast cell growth inhibition by clozapine, but were not able to repeat the results, see Figure 2.

Figure 2. Clozapine-induced yeast cell growth inhibition in the presence of H₂O₂ - Results of Dr. Abraham Kovoov's lab from the 24th of January, 2008



We first began by culturing the Y101 yeast strain in different media to determine which would produce the optimal yeast growth. YNB⁺ media containing the necessary amino acids (His, Leu, Lys, and Ura) did not allow for strong enough yeast growth. Spectrophotometer plate readings of the culture absorbance at 600 nm did not show yeast growth greater than 0.1 and there was no visible turbidity. However, synthetic complete (SC) media did result in visible yeast culture growth in 96-well plates with 100 mL of SC media in each well. YPD media was used to grow starting liquid cultures of the Y101 yeast strain from a frozen stock plate.

Once it was established that Y101 would grow properly in YPD and SC media, the correct amount of yeast for each reaction mixture had to be established. As variables were added to the reaction mixture the ideal yeast concentration was continuously changed or adjusted to produce optimal yeast growth in order to assay the affect of clozapine. Many aspects of the protocol were changed to result in different yeast concentrations throughout the trial and error experimentation with different concentrations of the five variables (yeast, clozapine, DMSO, H₂O₂, and HRP):

- The time for the YPD liquid culture to grow was adjusted from one to two days.
- The amount of YPD liquid media in the original liquid yeast culture varied from 1.5 mL to 5 mL.
- The amount of a 5 mL YPD overnight culture that was used varied from an entire 5 mL overnight culture to seven decreasing yeast concentrations produced by a 1:10 dilution series of a 5 mL overnight culture.
- At one point, two 5 mL YPD liquid cultures were spun down and washed and the resulting yeast cells were combined and brought back up in 1 mL of SC media. This liquid yeast culture was then used to produce seven decreasing concentrations of yeast in a 1:10 dilution series.
- Not only was the concentration of the liquid yeast culture added to the reaction mixture

manipulated, but the amount of the culture added also varied from 10 μL to 40 μL of yeast culture.

The concentrations of DMSO and H_2O_2 were also experimented with at length. Before clozapine was added to the reaction mixtures, it was important to determine the concentrations of DMSO and H_2O_2 that could be tolerated by different concentrations of yeast. DMSO is the necessary solvent for clozapine and would therefore have to be present in the reaction mixtures. H_2O_2 was hypothesized to have a role in the mechanism by which clozapine induces cell death in white blood cells, therefore some of the reaction mixtures would have to contain H_2O_2 to determine whether or not H_2O_2 is also necessary for clozapine-induced yeast cell death and/or yeast cellular growth inhibition. Both DMSO and H_2O_2 are toxic to yeast cells in their own right. Therefore, it was necessary to determine the proper concentration of DMSO, H_2O_2 , and yeast cells that would still result in appropriate yeast cell growth. Different combinations of decreasing yeast, DMSO, and H_2O_2 concentrations were tested to find the appropriate concentrations that would not result in overly strong cell growth (since the strongly growing cultures may simply grow too strong to show the effects of clozapine), but that would still result in cell growth sufficient enough to test the effect of clozapine on its growth. High concentrations of DMSO and H_2O_2 resulted in no cell growth even at high concentrations of starting yeast culture, indicating the importance of accounting for and controlling the toxic effects of DMSO and H_2O_2 . The DMSO amounts varied from 2.5 μL to 20 μL and H_2O_2 concentrations varied from 1% to $1 \times 10^{-10}\%$. Once yeast was growing in the presence of DMSO and H_2O_2 , clozapine could be added and cellular growth inhibition could be safely concluded to be resulting from clozapine and not the other toxic compounds alone (DMSO and H_2O_2).

When clozapine was added at varied concentrations of 5 μM to 20 μM , the variables of yeast concentration and H_2O_2 again had to be manipulated to result in yeast growth. The proper amount of DMSO to be tolerated by yeast cells had been determined to be 5 μL of DMSO. Therefore, the different clozapine concentrations were diluted with water so that all three concentrations resulted in a solution containing only 5 μL of DMSO. Yeast concentration was varied until yeast was growing properly in the control wells ('no clozapine' and 'no clozapine or H_2O_2 ') and growing to some extent in the reaction mixtures containing clozapine. Once it was established that 20 μL of a 2 mL two-day culture of YDP would result in the proper yeast concentration, this exact amount of yeast was added to each well in the 96-well plates thereafter, and no further manipulation of yeast concentration was necessary.

Next, we experimented to determine what concentrations of H_2O_2 would still allow for yeast cell growth but might also possibly allow for dosage dependent clozapine yeast cell growth inhibition. The concentrations of H_2O_2 and clozapine were still manipulated and many 96-well plates were created with different combinations of five decreasing concentrations of H_2O_2 and the increasing dosages of 0 μM , 5 μM , 10 μM and 20 μM clozapine. Also present in these experimental plates was the fifth variable: HRP. Up until this point, HRP had only been used in some of the trials to determine its effect, which was decreased cellular growth inhibition. However, in this phase of the project, where we were attempting to show dosage dependent clozapine-induced yeast cell growth inhibition by testing the different combinations of clozapine and H_2O_2 concentrations, these combinations were tested both with and without 2U HRP. Figure 3 represents an example of the experimental set-up in the plates used to

determine the proper H₂O₂ concentration that resulted in dosage dependent clozapine-induced cell growth inhibition of the Y101 yeast cultures, of the predetermined concentration.

Figure 3. Example of 96-well plate experimental set-up used to show clozapine-induced yeast cell growth inhibition

	0U HRP						2U HRP					
	0%	1%	0.10%	0.01%	0.001%	0.0001%	0%	1%	0.10%	0.01%	0.001%	0.0001%
	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂	H ₂ O ₂
0uM Clozapine												
5uM Clozapine												
10uM Clozapine												
20uM Clozapine												

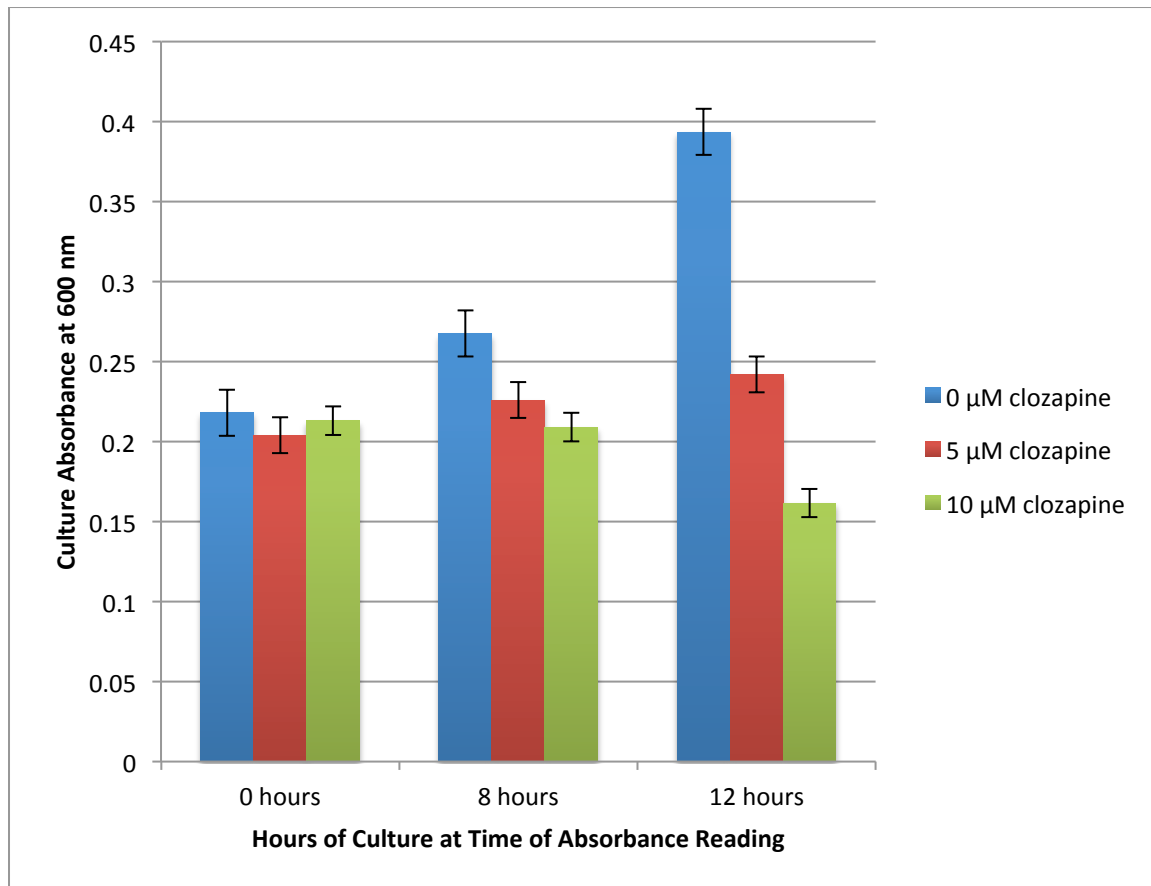
Results

Clozapine inhibits growth of yeast in a dose dependent manner

The conditions to study the effects of clozapine were optimized using a growth inhibition assay, performed in 96-well plates. Different combinations of varying concentrations of the four variables involved in the reaction (clozapine, DMSO, H₂O₂, and HRP) were used, and three different concentrations of yeast were tested. The assay was performed in 100 μL synthetic complete (SC) media to which 20 μL of two-day liquid yeast culture grown in 1.5 mL of YPD media was added. Clozapine, solvated in 5 μL of DMSO, was used in the assay at the different concentrations of 0 μM, 5 μM, 10 μM and 20 μM. Also, used in a successful assay was 1% H₂O₂.

11 trials resulted in culture absorbencies at 600 nm (A_{600}) that indicated dose dependent clozapine-induced yeast cell growth inhibition in the presence of 1% H₂O₂. Figure 4 is a representative trial with the duplicate well data averaged. Data presented in Figure 4 indicates a negative correlation between the concentration of clozapine and A_{600} , suggesting that clozapine was inhibiting the growth of yeast cells.

Figure 4. Represents the growth of yeast cells in the presence of increasing concentrations of clozapine; one representative trail with duplicates averaged



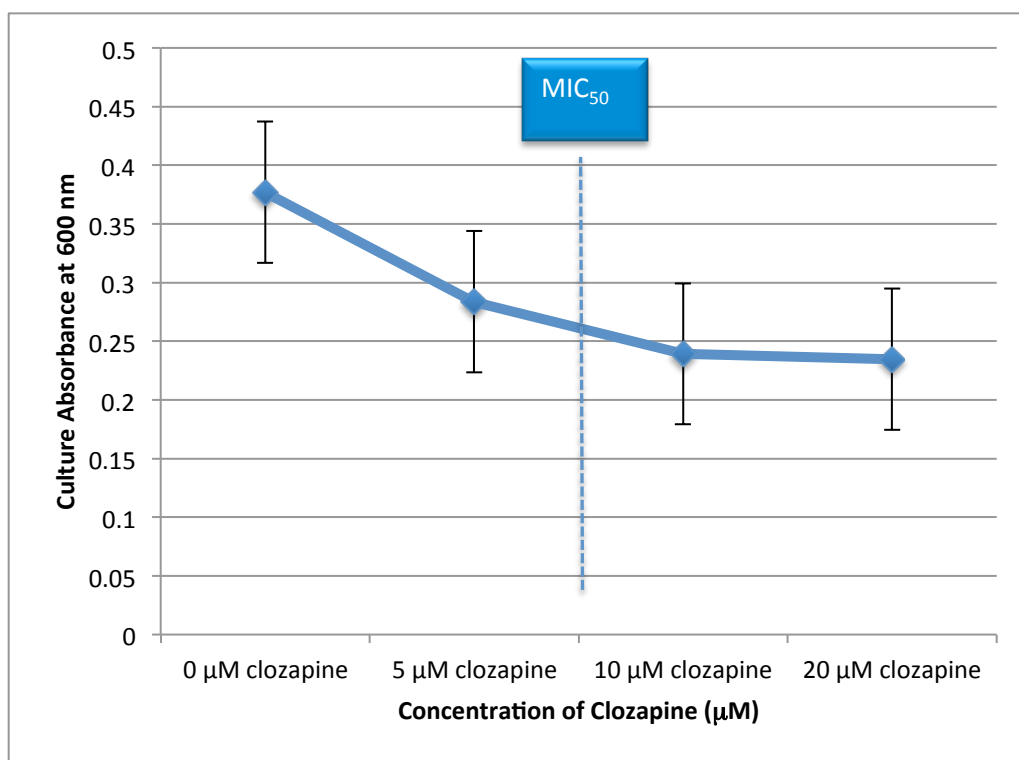
The data in Figure 4 shows that clozapine inhibits the growth of yeast at a concentration as low as 5 μM, and this effect is exaggerated after 12 hours. Furthermore, the growth inhibition phenotype is dose dependent. There is a striking decrease in A_{600} reading between 0 μM clozapine and 5 μM clozapine than between 5 μM clozapine and 10 μM clozapine. A_{600} was also measured at 20 μM clozapine, but there was no significant difference in growth inhibition between 10 μM clozapine and 20 μM clozapine (data not shown).

The estimated minimum inhibitory concentration of clozapine for *S. cerevisiae* is between 5 μM and 10 μM

The minimum inhibitory concentration (MIC_{50}) is defined as the concentration of clozapine that inhibits growth of 50% of the yeast cells. From this study we can estimate that the MIC_{50} for clozapine is between 5 μM and 10 μM. To determine an exact MIC_{50} we need to test more concentrations of clozapine between 5 μM and 10 μM. For the purpose of this study we noted that yeast cell growth inhibition started after eight hours and was significantly inhibited by 12 hours. Therefore, the minimum

inhibitory concentration was estimated from the culture absorbance readings at 600 nm taken after 12 hours of culture. Three 96-well plate trials were chosen to estimate the MIC₅₀, because they showed the clozapine-induced cell growth inhibition phenotype most clearly. Since each plate contained duplicates, this resulted in six culture absorbencies at each concentration of clozapine. Figure 5 represents a simplified version of the graph used to determine the MIC₅₀ of yeast cells, with the average of the six absorbencies at each clozapine concentration shown. The average culture absorbance for the six reproducibly repeated trial absorbencies at 600 nm and after zero hours of culture was determined to be 0.377. When this was divided in half it resulted in 0.189, which represents a cell culture at half the average starting density. This absorbency of 0.189 intersected the curves representing the different trials between the culture absorbencies for 5 μM clozapine and 10 μM clozapine, indicating that the MIC₅₀ is some concentration between 5 μM and 10 μM clozapine. Figure 5 shows the average cell density at each concentration of clozapine and the standard deviation of the averages. This graph also shows that the MIC₅₀ of yeast cells for clozapine falls between 5 μM and 10 μM clozapine.

Figure 5. Represents the average culture absorbance after 12 hours of incubation in the presence of increasing concentrations of clozapine; average of three reproducibly repeated trials



Discussion

The major contribution of this project was to establish that clozapine causes yeast cell growth inhibition of *S. cerevisiae*, and consequently that *S. cerevisiae* may be able to serve as a model for understanding white blood cell (WBC) loss. Assuming the mode of action of WBC loss is similar to yeast

cell growth inhibition, this assay can be used to screen the *S. cerevisiae* deletion library to identify genes and pathways involved in clozapine's mechanism of agranulocytosis. Clozapine causes a decrease in WBC count by actually killing white blood cells and not by inhibiting their growth. It would be significant to establish that clozapine also kills yeast cells, as it does white blood cells, and that the decreased culture absorbance is not simply due to yeast cell growth inhibition. Yeast cell death can be easily assayed using simple dyes that test cell viability.

Furthermore, performing this growth inhibition assay with other atypical antipsychotics, such as olanzapine, would aid in further establishing whether or not the interaction between yeast and clozapine can serve as a model for the interaction between yeast and white blood cells. If other atypical antipsychotics, which do not induce agranulocytosis, do cause dosage dependent atypical drug-induced yeast cell growth inhibition then this would mean that the yeast cell growth inhibition caused by clozapine might be due to a mechanism other than the mechanism that causes WBC death. Also, removing the yeast cell wall before performing the same assay we developed would make the model more similar to white blood cells, which do not have cell walls. It is impossible to know what compounding effects the yeast cell wall may be having on the assay.

We were able to develop a dependable assay for the effect of clozapine on yeast cell growth. This assay was successfully repeated 11 times, in which each time both duplicates showed normal growth without clozapine and inhibited growth after 12 hours in the presence of clozapine and H₂O₂. Future experiments attempting to elucidate the mechanism of clozapine-induced agranulocytosis using yeast as a model system, such as future *S. cerevisiae* genomic screens, will surely find this assay valuable. This study could only estimate the MIC₅₀ of clozapine for yeast cells to be between 5 and 10 μM. To determine the exact MIC₅₀ a range of concentrations of clozapine, between 5 and 10 μM, should be tested to focus the study. This will also give investigators a better understanding of what concentrations of clozapine with which to conduct the genomic screens in order to identify resistant and sensitive mutants. Future experiments should be done to further narrow the range of concentrations of clozapine that account for the MIC₅₀.

Finally, clozapine-induced cell growth inhibition was apparent only in the presence of H₂O₂ indicating that H₂O₂ is necessary for clozapine to inhibit cell growth. In the control group of reaction mixtures containing no H₂O₂, there was no inhibition of cell growth even at the highest concentration of clozapine. This is a significant observation, because we hypothesize that granulocytes produce H₂O₂ upon endocytosis of clozapine. The data supports our hypothesis of an immune dependent mechanism of action for clozapine-induced agranulocytosis. Further study will establish the exact nature of the interaction between clozapine and yeast and whether the interaction between clozapine and white blood cells is similar.

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